

Protective Role of Curcumin Against Arsenic Trioxide Toxicity During Gestation and Lactational Periods

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Abstract: Oxidative stress was induced by oral administration 0.4 mg/kg b.wt of arsenic trioxide (As_2O_3) during embryonic development in experimental mice. The level of liver arsenic concentration, lipid peroxidation, reduced glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) were determined in adult male Swiss Albino Mice. Hepatotoxicity was assessed by quantifying the aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP). Hepatoprotective efficacy of curcumin (7.5mg/kg b.wt) was evaluated by combination treatment with As_2O_3 . The theme of our study was to evaluate the efficacy of curcumin in combating arsenic induced hepatic oxidative stress, histopathological changes and the hepatic arsenic accumulation in mice model. Results revealed that As_2O_3 administration leads to the generation of reactive oxygen species (ROS), arsenic accumulation, serum marker enzymes release and decrease in antioxidant enzymes in liver. Retention of arsenic in liver caused increased level of lipid peroxidation with a concomitant decline in the glutathione dependant antioxidant enzymes and antiperoxidative enzymes. Curcumin treatment protected the liver from arsenic induced deterioration of antioxidant levels as well as oxidative stress. And also a significant decrease in hepatic arsenic accumulation and serum marker enzymes was observed. Histopathological examination revealed a protective effect of curcumin to liver tissue. From arsenic-induced toxic effects during embryonic development.

Key words: Embryonic Development • Arsenic Trioxide • Curcumin • Oxidative Stress • Arsenic Accumulation • Hepatoprotective.

INTRODUCTION

Arsenic is one of the most important global environmental toxicants and the human exposure comes mainly from consumption of drinking water contaminated with inorganic arsenic [1]. In clinical trials it is considered as a first choice cancer chemotherapeutic against certain leukemia and has potential against a variety of other cancers, including solid tumors [2]. Specifically, arsenic trioxide (As_2O_3) is used in the treatment of acute promyelocytic leukemia and it greatly improves the clinical outcome even in refractory or multiple relapsed cases [3]. But, toxic side effects of arsenicals are often a major concern, including the potential for fatal hepatotoxicity [4].

The liver is a major target organ for both arsenic metabolism and toxicity. Arsenic induced hepatic injury is

known to be exerted through excess production of reactive oxygen species, namely superoxide ($O_2^{\bullet-}$), hydroxyl ($\bullet OH$) and peroxy (ROO^{\bullet}) radicals and hydrogen peroxide (H_2O_2) [5]. The harmful expressions of arsenic are primarily due to an imbalance between pro-oxidant and antioxidant homeostasis in physiological system and also due to its fascination to bind sulfhydryl groups of proteins and thiols of glutathione (GSH) [6]. Thus, an agent able to reduce the toxic potential of arsenic in liver cells would clearly be a useful compound for arsenical chemotherapy.

Curcumin [1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6 heptadiene-3, 5-dione (diferuloylmethane)] is a naturally occurring polyphenolic compound derived from the root of *Curcuma longa* Linn. a perennial herb belonging to ginger family Zingiberaceae [7]. Curcumin has been shown to exhibit antioxidant, anti-inflammatory,

antimicrobial and anticarcinogenic activities [8]. Curcumin could effectively be considered a good candidate for cancer prevention when used alone and for cancer treatment in combination with other conventional therapies as it is able to target multiple signaling pathways implicated in this disease [9]. Thus, in the present study, curcumin was selected as the most potential natural compound to prevail over arsenic induced hepatotoxicity.

Arsenic exposure leads to the incidence of hepatotoxicity as manifested by increase in the levels of total bilirubin, alanine aminotransferase, aspartate aminotransferase and malondialdehyde [10]. Curcumin is well known as a multifunctional drug with anti-oxidative, anti-cancerous and anti-inflammatory activities [11]. More and more preclinical studies support the idea that curcumin, a plant-derived natural polyphenol, could be a promising anticancer drug. [12]. Hepatoprotective, antioxidant, antimutagenic and anticarcinogenic effects of curcumin have been shown by previous workers [13]. This study was designed to evaluate the protective role of curcumin upon As_2O_3 induced hepatotoxicity.

MATERIALS AND METHODS

Chemicals and Reagents: Arsenic trioxide, curcumin, sodium pyruvate, thiobarbituric acid and triton X-100, phenazine methosulphate, nitroblue tetrazolium (NBT) and bovine serum albumin were obtained from Sigma-Aldrich, Bangalore, India. L-aspartate, α -oxoglutarate, 2,4-dinitro phenyl hydrazine, nicotinamide adenine dinucleotide (Reduced), thiobarbituric acid, 5,5'-dithiobis-nitro benzoic acid, nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione, 1-chloro-2,4 dinitro benzene (CDNB) were purchased from Merck Specialties Pvt. Ltd., Mumbai, India. All other chemicals were purchased from Sisco Research Laboratories (SRL), India.

Animals: Twenty four male Swiss Albino mice weighing 30 g were purchased from the College of Veterinary Science, Prddatur, India and acclimatized for six days. All the animals were maintained under standard laboratory conditions of temperature ($23 \pm 1^\circ C$) and 12 hour light and dark cycles throughout the experimental period. The mice were provided with laboratory chow (Hindustan Lever Ltd., India) and tap water adlibitum. Experiments were conducted as per the guidelines of Institutional Animal Ethical Committee, Sri Venkateswara Veterinary University, Tirupati.

Experimental Protocol: Animals were divided into four groups of six mice each, a normal control group, a curcumin control which received 7.5 mg/kg b.wt of curcumin, one As_2O_3 (0.4 mg/kg b.wt) administered group and a combination group treated with 0.4 mg/kg b.wt of As_2O_3 and 7.5 mg/kg b.wt of curcumin. 0.2% DMSO solution was used as vehicle for curcumin administration. Experimental groups received this via oral intubation daily for gestational and lactational periods.

At the end of the experimental period animals were decapitated, blood was collected and centrifuged at 3000 rpm for 20 minutes; the clear serum obtained was used for the determination of marker enzymes. Liver was removed immediately, washed in ice cold 0.15M NaCl and blotted on a filter paper. Then the tissue was weighed and homogenized by using Teflon glass homogenizer (1/10th weight/volume) in ice cold tris-HCl buffer (0.2M, pH 7.4). The homogenate was centrifuged at 10000g for 20 min at $4^\circ C$ and the supernatant was used for the estimation of lipid peroxidation and various enzymatic and non enzymatic assays.

Serum Enzyme Analysis: The activities of lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were detected by kinetic method using the kit of Agappe Diagnostic Ltd., India. Activities of these serum enzymes were measured by using semi auto analyzer (RMS, India).

Detection of Arsenic Accumulation in Liver Tissue: Liver tissue of experimental mice were digested by thermal acid microwave digestion and diluted with double distilled water. Total arsenic deposition in liver tissue was analyzed by standard inductively coupled plasma-optical emission spectroscopy (Optima 2000 DV ICP-OES, Perkin Elmer, Inc. USA).

Tissue Analysis: Tissue catalase (CAT) activity was determined from the rate of decomposition of H_2O_2 [14]. Glutathione peroxidase (GPx) activity was determined by measuring the decrease in glutathione (GSH) content after incubating the sample in the presence of H_2O_2 and sodium nitrite [15]. Glutathione reductase (GR) activity was assayed by the method of Goldberg and Spooner [16]. The amount of NADPH consumed during GSSG (glutathione oxidised) reduction was used as the index of enzyme activity. Superoxide dismutase (SOD) activity was measured by the method of Kakkar *et al.* [17]. One unit was taken as the amount of enzyme that gave 50% inhibition of NBT reduction/mg protein. Glutathione-S-

transferase (GST) activity was determined from the rate of increase in conjugate formation between reduced glutathione and CDNB [18]. Reduced GSH was determined according to the method of Ellman [19] based on the formation of a yellow coloured complex with DTNB. Lipid peroxidation was measured as malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS), using 1'1'3'3' tetramethoxypropane as standard [20]. Protein content in the tissue was determined [21] using bovine serum albumin (BSA) as the standard.

Histopathology: Small sections of liver, fixed in 10% buffered formalin were processed for embedding in paraffin. Sections of 5-6 μ m were cut and stained with hematoxylin and eosin and examined for histopathological changes under the microscope (Motic AE 21, Germany). The microphotographs were taken using Moticam-1000 camera at original magnification of 100X.

Statistical Analysis: The results were analyzed using statistical programme SPSS/PC+, version 10 (SPSS Inc. Chicago, IL, USA). One way ANOVA was employed for comparison among the three groups. LSD post hoc multiple comparison test was used to determine significant difference among groups. $p < 0.05$ was considered significant.

RESULTS

Effect of Curcumin on Arsenic Trioxide Induced Hepatotoxicity: Figure 1A shows that treatment with As_2O_3 caused a significant ($p < 0.05$) increase in the concentration of arsenic in the liver tissue. Continuous administration of As_2O_3 for gestation and lactational periods of at a dose of 0.4mg/kg body weight caused arsenic deposition in liver. Co-treatment with curcumin significantly ($p < 0.05$) decreased the accumulation of arsenic.

Effect of Curcumin on Arsenic Trioxide Induced Changes in the Liver Marker Enzymes in Serum: Liver markers were monitored by estimating AST, ALT and ALP in the serum of mice. The diagnostic marker enzyme activities were significantly increased ($p < 0.05$) in arsenic treated mice, indicating hepatic dysfunction. Curcumin co-treatment exerted a reduction in the activity of AST (Fig. 1B) ALT (Fig. 1C) and ALP (Fig. 1D).

Effect of Curcumin on Arsenic Trioxide Induced Changes in the Liver Lipid Peroxidation and Antioxidant Enzyme Activity: The effect of curcumin on lipid peroxidation and the activity of antioxidant enzymes were outlined in the Table 1.

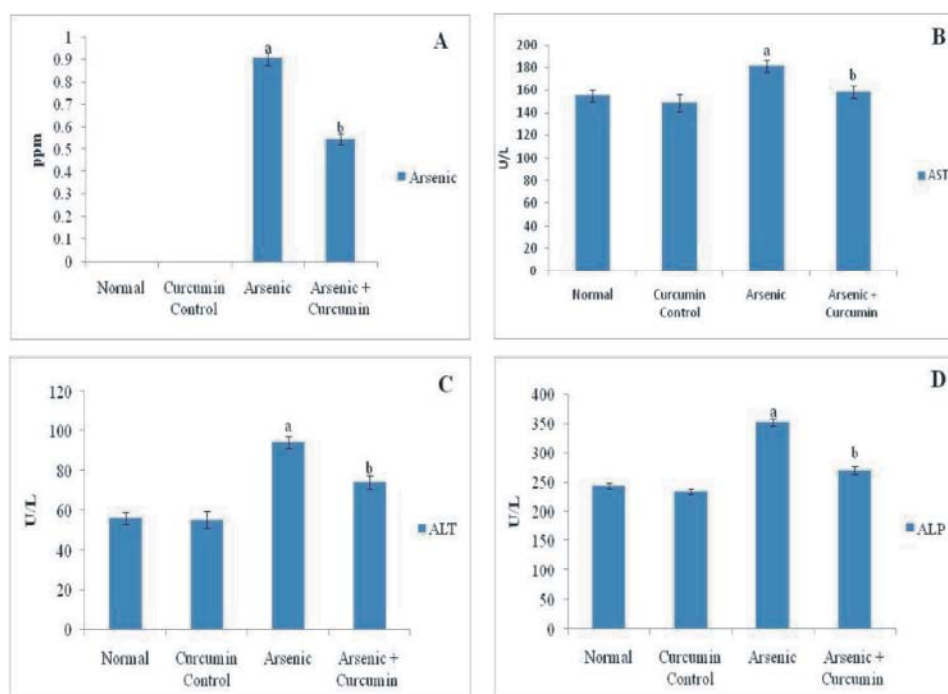


Fig. 1: Effect of curcumin on arsenic trioxide induced variations in arsenic accumulation and the liver marker enzymes in serum:(A)-residue of arsenic in hepatic tissue (B)- changes in levels of Aspartate aminotransferase (C) Alanine aminotransferase and (D) Alkaline phosphatase in different phases of toxicity and treatment

Table 1: Effect of curcumin on arsenic trioxide induced changes in lipid peroxidation and antioxidant status.

Parameters	Normal control	Arsenic	Arsenic + Curcumin
TBARS (nM/mg protein)	3.17±0.17	5.49±0.27 ^a	3.99±0.15 ^b
SOD (U/mg protein)	9.12±0.11	7.73±0.15 ^a	8.78±0.13 ^b
GST (μM of CDNB- GSH conjugate formed /min/mg protein)	1.24±0.08	0.52±0.09 ^a	1.09±0.1 ^b GPx
(ig of GSH consumed/min/mg protein)	11.26±0.31	8.18±0.33 ^a	10.82±0.42 ^b
CAT (μ moles of H ₂ O ₂ consumed /min /mg protein)	32.96±2.30	23.01±2.15 ^a	29.96±2.14 ^b
GSH (μM/g tissue)	52.78±7.5	34.88±5.8 ^a	47.31±4.71 ^b
GR (nmol of NADPH oxidised/min/mg protein)	0.66±0.03	0.40±0.02 ^a	0.52±0.02 ^b

Data represented as mean ±SD, n=6. ^ap<0.05 versus normal control, ^bp<0.05 versus As₂O₃ treated groups.

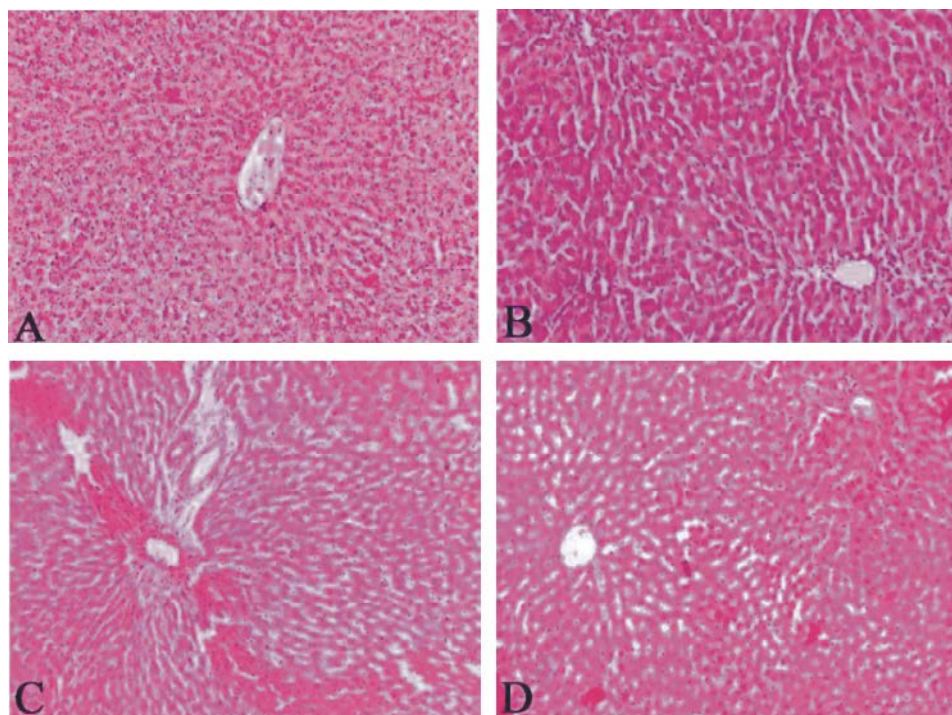


Fig. 2: Histopathology of mice liver tissue showing (A) Normal control group. (B) Curcumin control group, (C) As₂O₃ hepatotoxic effect (D) As₂O₃ + Curcumin towards recovery.

Thiobarbituric acid assay was used to measure the extent of lipid peroxidation induced by As₂O₃ in liver of mice. Results indicated that TBARS level was significantly (p<0.05) increased in liver of mice treated with As₂O₃. Co-treatment with curcumin caused significant (p<0.05) decrease in liver TBARS compared to the As₂O₃-treated mice.

A significant (p<0.05) decline in the level of GSH was noticed in liver tissue of arsenic treated mice as compared to controls. As₂O₃ administered mice showed significant decrease (p<0.05) in the activity of SOD, CAT, Gpx, GST and GR in hepatic tissue. Co-treatment with curcumin exhibited significant increase in the GSH levels compared to As₂O₃ mice. The curcumin treatment showed significant

(p<0.05) elevation in the antioxidant enzymes activity (Table 1).

Effect of Curcumin on Arsenic Trioxide Induced Changes in the Liver Histopathology:

Histopathological examination of hepatic tissue showed marked abnormalities such as. moderate sinusoidal dilation, hemorrhage, focal necrosis and cholangiofibrosis in arsenic treated mice (Fig.2C). Structural abnormalities in As₂O₃ treated liver was significantly prevented by co-treatment with curcumin. Curcumin co-treated mice showed only mild sinusoidal dilation; no other significant pathological changes were observed (Fig. 2D). Liver tissue of normal control (Fig. 2A) and curcumin control (Fig. 2B) showed normal hepatocellular morphology.

DISCUSSION

Curcumin or diferuloylmethane is a polyphenolic molecule derived from the rhizome of the plant *Curcuma longa* [22]. The present study demonstrates the curative potential of curcumin by reversing As_2O_3 induced hepatic damage. Arsenic has been showing stunning efficacy in the treatment of acute promyelocytic leukemia [2]. However, inorganic arsenic can have profound toxic effects even following short-term use. For instance, potentially fatal hepatic toxicity has been reported in a subset of patients receiving arsenic chemotherapy [4]. The dose of curcumin (15 mg/kg body weight) was selected from a recent study [23]. The dose of As_2O_3 was 4 mg/kg body weight daily, which has been shown to produce plasma concentration of arsenic within the near range of those presented in arsenic treated APL patients [24].

As_2O_3 treatment in this experiment resulted in a significant increase in the level of TBARS in hepatic tissue. This elevation might be because of the lower level of SH-groups and antioxidant enzymes also observed in this study. Generation of large amount of reactive oxygen species (ROS) due to arsenic toxicity can overwhelm the antioxidant defense mechanism and damage cellular ingredients such as lipids, proteins and DNA; this in turn can impair cellular structure and function. Curcumin a known scavenger of free radicals, when administered in the current study, efficiently lowered the peroxidation levels thus protecting tissues from oxidative stress. In agreement with Kalpana *et al.* [25], data obtained in the present study also showed that curcumin significantly decreased the levels of TBARS.

As_2O_3 treatment caused arsenic accumulation in liver cells. The increased concentration of arsenic in liver may be due to an insult in the detoxification mechanism. The liver is also the major site of arsenic methylation, which is catalyzed by arsenic methyl-transferase using S-adenosyl methionine (SAM) as the substrate [26]. This enzyme catalyzes the transfer of a methyl group from SAM to trivalent arsenic producing methylated and dimethylated arsenic compounds. Arsenic is methylated to monomethyl arsenic acid (MMA) and finally to dimethyl arsenic acid (DMA) [27]. Our study also showed that the exposure to As_2O_3 significantly increased ROS production and enhanced oxidative stress in hepatocytes. One of the most important arsenic detoxification mechanisms is the glutathione (GSH) system. Oxidative stress-mediated hepatotoxic effect of arsenic is mainly due to the depletion of GSH in liver. Depletion of hepatic GSH facilitates accumulation of arsenic in the liver and thus causes

oxidative stress [28]. Therefore, curcumin-induced increased GSH level during exposure to toxic electrophiles generated by arsenic as well as its rapid elimination/excretion from the body play an important role in decreasing oxidative stress. Hepatocellular arsenic deposition may be the prime reason for liver toxicity. Curcumin treatment significantly reduced the arsenic accumulation and structural abnormalities in hepatic tissue.

In the present study, treatment with As_2O_3 caused significant increase in the activities of hepatic marker enzymes in serum; this may be due to the leakage of the enzymes to the blood stream. The increase in the activities of these enzymes in plasma indicates liver damage and alteration in liver function. Treatment with curcumin significantly decreased the activities of these enzymes in serum suggesting that it offers protection by preserving the structural integrity of the cell. Administration of curcumin preserved the integrity of the hepatocellular membrane. Our results were in agreement with Yu *et al.* [29] who demonstrated that curcumin prominently reduced tissue injury as well as ALT and AST activity. The leakage of enzymes because of liver injury was prevented by the liver cell membrane stabilizing action of curcumin.

A significant reduction in the activities of glutathione dependant antioxidant enzymes (GPx and GST) and antiperoxidative enzymes (SOD and CAT) with a concomitant decline in the level of GSH level was observed in the liver tissue of As_2O_3 treated mice. GST plays a key role in cellular detoxification by catalyzing the reaction of glutathione with toxicants to form an S substituted glutathione [30]. SOD is the first antioxidant enzyme to deal with oxy-radicals by accelerating the dismutation of superoxide to hydrogen peroxide, while CAT is a peroxisomal heme protein that catalyses the removal of hydrogen peroxide formed during the reaction catalyzed by SOD. Thus, SOD and CAT acts mutually supportive anti oxidative enzymes, which provide protective defense against reactive oxygen species [31]. Reduced activity of CAT after exposure to As_2O_3 in the present finding could be correlated to increased generation of H_2O_2 . Inorganic arsenic exposure results in the generation of ROS in various cellular systems and its production has been proposed as one of the early biological events on arsenic-related carcinogenic process [32].

Curcumin has two o-methoxy phenolic OH groups attached to the α -diketone moiety having methylene CH_2 group. It is believed that the H abstractions from these groups are responsible for the remarkable antioxidant

activity of curcumin. The free radical scavenging activity of curcumin can arise by the resonance stabilization of its radicals from two phenolic OH groups (mainly) or from the CH₂ group of the α -diketone moiety [33]. Therefore, curcumin not only is a phenolic antioxidant that mostly donates H atoms from the phenolic groups, but also is a α -diketone radical chain-breaking substance that can give H atom from methylene CH₂. Treatment with curcumin significantly reduced intracellular ROS production by increasing the activity of GSH generating enzyme GR and ROS scavenging enzymes (GST, GPx, SOD and CAT) in hepatic tissue.

Curcumin is known as antioxidant and anti-inflammatory properties. It is the free radical scavenger and inhibited lipid peroxidation products [34, 35]. The protective mechanism of curcumin may due to the strong antioxidant property i.e. it helped for the healing of hepatic parenchyma and regeneration of hepatocytes. Histopathology was also consistent with As₂O₃ induced damage in the concerned organ. Treatment by curcumin restored normal hepatic histopathology as well as various diagnostic biochemical variables towards normal indicating reversal of As₂O₃ induced hepatotoxicity and confirming the free radical scavenging property of curcumin.

In conclusion, the present study shows that curcumin treatment mitigates arsenic intoxication-induced oxidative damage, which could be due its antioxidant nature that combines free radical scavenging and metal chelating properties. As a single agent arsenic caused toxic effects in hepatic tissue but along with curcumin the side effects were significantly reduced. It can be summarized that curcumin could be a potential antioxidant against the oxidative stress generated by As₂O₃. However, it is suggested that curcumin might be a useful hepatoprotective agent in cancer therapy along with standard chemotherapeutic drug As₂O₃.

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